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(19)(CA) **APPLICATION FOR CANADIAN PATENT** (12)

(54) Synchronized Donor Cells for Bovine Nuclear Transfer

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SYNCHRONIZED DONOR CELLS FOR BOVINE NUCLEAR TRANSFER

Abstract

A method of multiplying bovine embryos by nuclear transfer includes the steps of synchronizing donor embryonic cells at a cell cycle phase using a cell cycle inhibitor, releasing the cells from inhibition, and allowing the cells to progress into the S-phase of the cell cycle before fusion to recipient oocytes. In a preferred process, the recipient oocyte is activated before fusion such that both donor and recipient are in S-phase when fusion occurs. More preferably, the cell cycles of both donor and recipient are coordinated before fusion such that both donor and recipient would re-enter M-phase at about the same time. The method is intended to increase the percentage of viable bovine nuclear transfer embryos to a percentage higher than can be obtained without synchronization of donor nuclei.

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REFERENCE NO. 01-8273

SYNCHRONIZED DONOR CELLS FOR BOVINE NUCLEAR TRANSFER

Field of the Invention

The present invention is generally directed to a process for multiplying bovine embryos, and is specifically directed to an improved process for synchronizing the cell cycle stage of donor embryonic cells and recipient oocytes so that the overall efficiency of the bovine embryo multiplication process is improved.

Background of the Invention

Embryo multiplication by nuclear transfer involves the transplantation of the living nuclei from embryonic cells, or the whole embryonic cells themselves, into recipient cells, typically unfertilized eggs after which the donor and recipient are fused. Such transfers are made in order to increase the number of genetically identical embryos which can be obtained from elite genetic stock. Once a fertilized embryo has reached a cleavage stage of having at least two cells, it becomes practical to transfer the nuclei from such cells, or the entire cells themselves, into recipient oocytes which have been enucleated, to thereby create multiple genetically identical embryos from such fusions. By allowing each of the fused nuclear transfer embryos to develop to a multi-cell stage, and then repeating the nuclear transplantation procedure, large numbers of genetically identical nuclear transfer embryos can be created from an original donor embryo. While blastomeres isolated from pre-gastrulation embryos have been most widely used as a source of donor

nuclei, other sources of donor nuclei include bovine embryonic stem cells and embryos from oocytes that have been matured, fertilized, and cultured in vitro. A limitation on the commercial use of this process, as practiced to date, arises from the fact that there are certain inefficiencies in the nuclear transfer process. Not all of the nuclear fusions created result in viable embryos. Not all viable embryos created by nuclear fusion turn out to be capable of creating a viable pregnancy in the cow resulting in a live calf. Accordingly, effort is currently being directed toward optimizing the efficiencies at each step in the procedure, so as to make the overall procedure more economically practical.

The techniques of bovine nuclear transplantation are generally described in two U.S. patents, U.S. Patent No. 4,994,384 (Prather et al.) and Patent No. 5,057,420 (Massey), both of which describe procedures for the serial multiplication of bovine embryos. In the techniques described in each of those patents, oocytes are recovered from the ovaries or reproductive tract of cows. The oocytes are selected for proper stage, and then are enucleated by physical aspiration through a transfer pipette, leaving an enucleated oocyte which still retains its external membranes. Synchronously, a donor embryo of the proper cell staging, typically at the cleavage or morula stage, is manipulated so that one or more cells or blastomeres are removed from the embryo. The donor cell which, of course, includes its nucleus, is then inserted into the perivitelline space of the recipient oocyte. An electrical pulse is then applied to fuse the membranes of the donor cell and the recipient oocyte, thus creating an activated, fused single cell embryo. That single cell nuclear transfer embryo can then be cultured either in vitro or in the oviduct of a mammal, until a stage in which it can be implanted into a recipient cow. A significant number of the fused embryos will retain viability, can be transplanted surgically or non-surgically into the uteri of cattle, and will result in live births of genetically identical calves.

In past nuclear transplantation procedures, individual cells within the same donor embryo were not synchronized with respect to the cell cycle, beyond the four-cell stage. Thus, it has only been at random that a particular donor blastomere would be at the correct cell cycle stage to achieve optimal development after nuclear transfer. What is lacking in the art is both knowledge as to which cell cycle stages are most appropriate and a method for manipulating and synchronizing the cell cycle stage of donor embryonic cells.

Several reports of efforts to optimize the cell cycle stage of donor cell nuclei in other mammals have been reported. Collas, P., et al., 46 Biol. Reprod. 492-500 (1992) have demonstrated that donor nuclei in early stages of the rabbit embryo cell cycle show enhanced ability to fuse into embryos that will mature to the blastocyst stage. Kono, et al., 37 Theriogenology 239 (1992) demonstrated that nuclear transfer mouse embryos derived from fusions using synchronized late-stage murine blastomeres were better able to develop to the blastocyst stage than embryos derived from blastomeres of other stages.

Summary of the Invention

The present invention is summarized in that a process for bovine nuclear transplantation includes the steps of (1) ensuring that a majority of the donor bovine embryonic cells are in metaphase, (2) allowing the embryonic cells sufficient time post-metaphase to progress into the S-phase of the cell cycle, (3) fusing individual donor embryonic cells with recipient oocytes, (4) culturing the nuclear-transferred fusion cells to the blastocyst stage, and (5) transferring blastocysts to recipient females to produce cloned calves. In an especially preferred embodiment of the invention, the donor embryonic cell and recipient oocyte are synchronized to each other, in that at the time of fusion both are in S-phase and both are expected to reach the next M-phase at approximately the same time.

It is an object of the present invention to further develop and define a step in a bovine nuclear transplantation process so as to increase the overall efficiency of the process.

It is an objective of the present invention to aid in the overall efficiency of bovine transplantation and multiplication processes, so as to result in greater numbers of viable pregnancies and multiplied genetically identical calves.

It is an object of the present invention to increase the percentage of nuclear transfer embryos that mature to the blastocyst stage.

Other objects, advantages, and features of the present invention will become apparent from the following specification.

Brief Description of the Drawings

Fig. 1 schematically depicts the donor cell cycle, indicating the time of removal of the synchronizing agent, the time of fusion and the time until next M-phase.

Fig. 2 schematically depicts the recipient oocyte cell cycle, indicating activation time, fusion time, and time until next M-phase.

Description of the Preferred Embodiment

In accordance with the present invention, a bovine embryonic cell destined to be the source of a donor nucleus in a nuclear transfer (NT) procedure is subjected to synchronization in S-phase prior to nuclear transplantation. In addition, fusion of a recipient oocyte and a donor embryonic cell yields a higher percentage of viable NT embryos if both are in S-phase and if both will next enter M-phase at approximately the same time. The present invention provides a method for achieving such synchrony between donor and recipient.

The embryonic donor cells useful in the method of the present invention may be obtained in a variety of ways. Embryonic cells are often obtained by flushing embryos from surgically recovered oviducts or may be non-

surgically flushed from the uterus in manners known to the art. Embryonic donor cells may also be obtained from *in vitro* maturation/*in vitro* fertilization/*in vitro* culture (IVM/IVF/IVC) procedures. In addition, embryonic stem cells cultured and maintained *in vitro* may also be used as donor cells in the bovine multiplication procedure of the present invention. Totipotent bovine embryonic stem cells have been cultured to the blastocyst stage, then implanted into recipient cows. The advent of systems to create and culture bovine stem cells permits the serial culture of large numbers of genetically identical donor cells. See PCT Published Patent Application No. WO 90/03432. In addition, bovine embryonic stem cells have been used successfully as donor cells in nuclear transplant experiments, yielding fetuses that have developed to at least the 6th month of gestation. In the present application, the term "embryonic cell" refers to any bovine embryonic cell that may be used as a donor cell in nuclear transfers, notably those cells derived from bovine embryos, from IVM/IVF/IVC procedures, and from bovine embryonic stem cell cultures. Each cell type is useful as a donor cell in the method of the present invention.

The synchronization step is most preferably done by culturing the embryonic donor cell in a culture medium containing an optimal concentration of a cell cycle inhibitor for a period of time sufficient to synchronize a majority of the embryonic cells in a particular mitotic phase, such as metaphase. The cell cycle inhibitor may be any agent that reversibly blocks the cell cycle at a defined phase, such as a drug that blocks the cell in metaphase by inhibiting the formation of spindle microtubules.

Cell-cycle-inhibited embryonic donor cells, blocked at a defined cell cycle phase, are subsequently released from the cell cycle block by removing the inhibitor from the culture medium. The donor cells are then allowed to progress through the cell cycle into S-phase to yield, after NT fusion, the greatest percentage increase of NT embryos maturing to the blastocyst stage when compared to

NT embryos produced from fusions of non-synchronized donor nuclei.

Less preferably, one may avoid the use of cell-cycle-inhibiting drugs by observing the embryonic cell division processes and noting when the embryos are in metaphase. After visually observing cell division, it should then be possible to culture the embryonic cells for a sufficient length of time to assure entry into the preferred cell cycle phase, as described below. However, this second approach lacks the definitive and repetitive staging accuracy of the cell cycle inhibitors and is less preferred.

It is herein demonstrated that the greatest percentage increase in blastocyst stage embryos is obtained when the donor embryonic cells are in the cell cycle phase commonly referred to as S-phase. In this specification, S-phase is also referred to as mid-stage, since it is in the middle of the cell cycle. S-phase is characterized by DNA synthesis, also referred to as DNA replication. One complete cell cycle takes approximately 20 hours in pre-gastrulation bovine embryos and other embryonic cells. S-phase occupies the bulk of that 20 hour cycle. While the precise beginning and end points of S-phase are imprecisely defined, bovine embryos are known to be in S-phase from about five hours after release from metaphase until about five hours before returning to metaphase. S-phase is preceded by G1-phase (herein, early-stage), during which the cell prepares for DNA synthesis, and is followed by G2-phase (herein, late-stage), during which the cell prepares for mitotic division of M-phase. In M-phase, chromosomes condense and prepare to separate into the two daughter cells. During the metaphase portion of the M-phase, the chromosomes line up at the metaphase plate poised to be distributed between the daughter cells.

The synchronized embryonic cells for use within the present invention are intended for a protocol of bovine embryo multiplication through nuclear transplant. It is appropriate therefore, at this juncture, to briefly

describe the overall bovine embryo multiplication procedure. Bovine ovaries are collected at the slaughter house, and are maintained in physiological saline for transportation from the slaughter house to the laboratory. Follicles ranging in size from 2 to 10 mm in diameter are then aspirated from the bovine ovaries. The immature oocytes contained within the follicular fluid are removed, buffered with HEPES, and washed in hamster embryo culture medium (HECM), described in Seshagare et al., 40 Biol. Reprod., 599-606 (1989), and then placed into drops of maturation medium consisting of 50 µl of tissue culture medium (TCM) 199 containing 10% fetal calf serum with appropriate gonadotropins, luteinizing hormone (LH) and/or follicle stimulating hormone (FSH), and estradiol under a layer of lightweight paraffin or silicon oil at 39°C. After twenty hours in the maturation media, the in vitro-matured oocytes are removed and placed in HECM containing 1 mg/ml of hyaluronidase. The cumulus cells are removed from the oocytes by repeated pipetting through very fine-bore pipettes. The stripped oocytes are screened for polar bodies, which are indicators of metaphase II. The selected metaphase II oocytes are then used further in the transplantation and multiplication procedures.

The oocyte enucleation portion of the cloning procedure is described in U.S. Patent No. 4,994,384, the specification of which is hereby incorporated by reference. Briefly, the metaphase II oocytes are either placed in HECM containing 7.5 micrograms per milliliter cytochalasin B for immediate enucleation or are placed in CRlaa plus 10% estrus cow serum, and are enucleated later, preferably sixteen to eighteen hours later. CRlaa medium contains 114 mM NaCl, 3.1 mM KCl, 25 mM $NaHCO_3$, 0.4 mM Sodium Pyruvate, 5 mM Hemicalcium Lactate, 1 mM Glutamine, 1 ml/100 ml MEM amino acids, 2 ml/100 ml BME amino acids, and 50 mg/ml Gentamycin.

Enucleation is accomplished using a micropipette to remove the polar body and the surrounding cytoplasm. The oocyte can then be screened to determine which oocytes

have been successfully enucleated. This screening is successfully done by staining the oocytes with 1 microgram per milliliter 33342 Hoechst dye in HECM, and then viewing the oocytes under ultraviolet irradiation for less than 10 seconds. The oocytes that have been successfully enucleated can then be placed in culture media, preferably CR1aa plus 10% estrus cow serum. After enucleation, an embryonic donor cell is placed in the perivitelline space, by the method also described in the above-identified Patent No. 4,994,384.

For oocytes matured in vitro, the nuclear transfer process is normally performed between 20 and 46 hours after the oocyte was first placed in the maturation medium. After the blastomere cell is transferred into the perivitelline space of the recipient oocyte, the two cells are fused together by electrofusion. The cells to be fused are placed between two electrodes which are 500μ apart, referred to as the fusion chamber, which contain Zimmerman's fusion medium. Up until the point of fusion the donor cells and the recipient cells are maintained at or below 39°C, a physiological temperature.

After fusion, the NT embryos thus created are then placed in CR1aa medium plus 10% estrus cow serum at 39°C for 5 to 9 days. A detailed description of a process for culturing such NT embryos in vitro is set forth in U.S. Patent No. 5,096,822. At the end of this culture period, the embryos can be judged for developmental rates. The embryos judged to have developed normally, either into morulae or blastocysts, can be transferred into recipient animals, and an offspring may be obtained after a normal pregnancy.

The above brief description of the embryo multiplication and nuclear transplant process has been altered in the inventive method described herein only in the preparation of the donor embryonic cells and recipient oocytes. It has been found that fusions performed with donor cells synchronized in S-phase of the cell cycle yield a greater percentage of blastocysts for implantation than either non-synchronized donor embryonic cells or

embryonic cells synchronized in early (e.g., G1-phase) or late (e.g., G2-phase) cell cycle stages. In a preferred fusion method, the donor embryonic cells and recipient oocytes are both in S-phase at the time of fusion. In a most preferred fusion method, the donor cells and recipient oocytes are synchronized in that fusion takes place when the time-until-next-M-phase for both donor and recipient cells is approximately equal. In other words, it has herein been found that, a salient indicator of success appears to be the time remaining in the cell cycle until the next entry into M-phase, rather than the elapsed time since the previous M-phase. It is believed by the inventors that by synchronizing both donor and recipient cell cycles to each other during pre-fusion DNA synthesis, the cytoplasm and nucleus of post-fusion NT cell are better able to coordinately proceed into G2- and M-phases. In summary, if the donor cell is in S-phase, and also, preferably, if the recipient cell is at the same part of S-phase as the donor, th developmental success rate of blastocyst development in NT fusion embryos will be better than that of unsynchronized fusion embryos.

By performing routine preliminary experiments on the donor embryonic cells and activated recipient oocytes, one may easily determine the average cell cycle duration of each, using techniques that have been described. Determination of time-until-next-M-phase is depicted for donors and recipients respectively in Figs. 1 and 2. For synchronized embryonic donor cells, the time-until-next-M-phase is simply the cell cycle duration minus the time between removal of the cell cycle inhibitor and fusion. For oocytes matured and activated <u>in vitro</u>, the time-until-next-M-phase is the estimated cell cycle duration minus the time between activation and fusion. Figs. 1 and 2 are not meant to suggest that the cell cycle duration of embryonic cells and oocytes are identical. Rather, the figures demonstrate how to determine the time-until-next-M-phase of either cell type. It is known that the cell cycle duration of oocytes matured <u>in vitro</u> decreases with increasing maturation time.

Oocytes used as recipient cells are developmentally blocked at metaphase until activated. Activation occurs in vivo at fertilization or in vitro, by a number of methods. After activation by any method, oocytes progress through the cell cycle toward a cell division in the next M-phase. Of course, haploid oocytes cannot divide into daughter cells having full complements of chromosomes. Haploid oocytes must receive male chromosomes either by fertilization or by nuclear transfer from a donor cell.

The donor cell synchronization process of the present invention begins by arresting the embryonic cell cycle at a defined phase of the cell cycle, such as metaphase, using a cell cycle inhibitor such as Demecolcine or Nocodazole. Removal of the inhibitor is followed by a waiting period of experimentally-determined length sufficient to allow the synchronized blastomeres to advance to the mid-stage of the cell cycle. This process yields NT donor cells which, when fused to oocytes and allowed to develop in vitro, demonstrate increased viability typical of the present invention.

Any cell cycle inhibitor that reversibly arrests the cell cycle at a predictable cell cycle phase and that, upon its removal, allows the cell cycle to proceed to the S-phase is envisioned to fall within the scope of the present invention. The optimal cell cycle inhibitor concentration for synchronizing the cells may be easily determined by titrating the concentration used to treat bovine embryos and choosing that which yields the highest percentage of embryo cells synchronized at the desired phase. Similarly, one can experimentally determine the appropriate time to S-phase by fusing post-inhibition donor cells to recipient oocytes after various waiting periods and then observing which waiting period yields the highest percentage of subsequent blastocyst formation. Alternatively, one may chart the incorporation of radiolabelled nucleotides into DNA to determine when S-phase begins.

Example 1

Synchronization of bovine donor embryo cells using Demecolcine

Demecolcine is a microtubule inhibitor known to arrest the development of mammalian cells in the metaphase of the cell cycle. To determine the concentration at which the highest percentage of bovine embryo cells became synchronized in metaphase after exposure to Demecolcine, pre-gastrulation bovine embryos were cultured in medium containing the three Demecolcine concentrations shown in Table 1. After 12-18 hours in medium containing Demecolcine, the number of embryo cells arrested at the metaphase stage was determined using a nuclear staining assay. Nuclear material in embryo cells is visualized by staining the cells with a nuclear dye, such as Hoechst 33342 at 1 µg/ml in HECM, and then viewing the embryo cells under ultraviolet irradiation for less than 10 seconds. The percentage of embryo cells synchronized in metaphase varied with the concentration of Demecolcine used to arrest the cell cycle, as shown in Table 1.

TABLE 1

Demecolcine concentration (µg/ml)	Number of Embryos	% of cells synchronized in metaphase
0.5	5	60
0.1	20	48
0.05	10	73

Table 1 indicates that preferably 0.05 µg/ml of Demecolcine be used to arrest the development of bovine embryo cells in metaphase. Accordingly, bovine embryos were incubated in 0.05 µg/ml of Demecolcine for 12-18 hours. After the Demecolcine incubation period, the embryos were washed and placed back into culture medium lacking the Demecolcine cell cycle inhibitor. Freed from the cell cycle block, the synchronized embryos then progressed into the cell cycle.

At various times after removal of the Demecolcine block, embryos were harvested and individual blastomeres were removed for use in nuclear transfers to determine the optimal post-metaphase cell cycle stage for a donor blastomere. Blastomeres from embryos were harvested at 4-8 hours post-metaphase (hereinafter, early-stage), at 8-14 hours post-metaphase (mid-stage), or at 15-20 hours post-metaphase (late-stage). At 8-14 hours post-metaphase, the majority of donor blastomeres were in S-phase. As a control, blastomeres from non-synchronized embryos handled in parallel were used for transfer experiments with the early-, mid-, and late-stage synchronized blastomeres.

Recipient oocytes removed from the follicles were matured *in vitro* for 20-24 hours prior to selection of the oocytes for metaphase II staging. The selected metaphase II oocytes were then cultured (or matured) for an additional 14-24 hour period at physiological temperatures (about 39°C).

All nuclear transfers were performed using entire blastomere cells. The NT embryos were cultured *in vitro*, as described in published PCT patent application WO 90/13627, prior to screening for blastocyst development.

Table 2 demonstrates the significant increase in the percentage of blastocysts that develop from NT embryos when the donor blastomeres have been synchronized and then allowed to progress to the mid-stage of the cell cycle (8-14 hours post-metaphase). In contrast, blastomeres that have progressed from metaphase to the early- or late-stages of the cell cycle show either a decreased ability, or insignificantly increased ability, to develop to the blastocyst stage when compared to non-synchronized donor blastomeres.

TABLE 2

Percentage of Blastocysts (Blastocysts Obtained/Total NT Embryos)		
	Synchronized Donor Blastomeres	Un-synchronized Donor Blastomeres
Early	0% (0/140)	5% (11/235)
Mid	14% (49/362)	7% (25/339)
Late	9% (30/348)	6% (27/461)

Blastocysts obtained after nuclear transplant of mid-stage blastomeres were transferred to recipient cows. Of 10 cloned calves born after transfer of NT blastocysts to maternal animals, none had either high birth weight or congenital anomalies often associated with calves produced by nuclear transplant techniques. Typically 25% of all nuclear transplant calves exhibit either high birth weight or congenital anomaly have been reported. The data of Example 1 suggest that by synchronizing donor blastomeres to the mid-stage of the cell cycle certain, possibly genetic, factors which lead to physical problems with nuclear transplant calves may be avoided.

Example 2

Synchronization of Nocodazole-treated Bovine Embryos and Cold-Culture-Activated Recipient Oocytes Matured for 42 Hours In Vitro

Using a protocol similar to that of Example 1, a second cell cycle inhibitor was employed to produce donor cells which, when fused to recipient oocytes, yielded embryos that developed to the blastocyst stage at an increased frequency relative to non-synchronized donor cells.

The cell cycle inhibitor Nocodazole, which arrests the mammalian cell cycle in metaphase, was tested at 0.1, 1.0, and 10 . concentration in culture medium as in Example 1 for its ability to optimally synchronize the cells of bovine embryos. It was determined that 1.0 μM treatment with Nocodazole for 12-18 hours proved best. As in Example 1, bovine embryos were synchronized in metaphase with 1.0 μM Nocodazole. Subsequent removal of the inhibitor from the culture medium allowed the embryos to progress through the cell cycle. At mid-stage (8-14 hours post-metaphase), blastomeres were harvested and used as fusion partners for recipient oocytes. At 8-14 hours post-metaphase, a majority of the blastomeres were in S-phase.

Oocytes used as recipients in Example 2, were prepared by culturing the recipient oocytes at a temperature in the range of 24-26°C during the maturation period. This cold culture step, relative to the normal bovine physiological temperature of 39°C (used in Example 1), has been shown to increase the survival of developing NT embryos to the blastocyst stage.

Cold culture is believed to serve as a form of pre-fusion activation of the recipient oocyte. Cold culture releases the oocyte cell cycle from the M-phase block that is typically observed in unactivated oocytes. Activation methods other than cold culture are also useful for increasing efficiency of the protocol. In vivo, oocytes are activated at the time of fertilization. Whatever the cause of the activation phenomenon, it has been used herein to increase the efficiency of a practical bovine embryo multiplication protocol.

Cold culture may be performed at any temperature significantly below physiological temperatures (e.g., below 30°C and above freezing.) Further, the duration of cold culture may vary from as little as approximately 2 hours to 24 hours or more. All recipient oocytes in Example 2 were cold shock cultured, whether they were recipients of Nocodazole-treated embryonic cells or control cells.

In Example 2, recipient oocytes were matured for approximately 42 hours before cold culture activation. Oocytes that have matured for this length of time have a cell cycle of about 10 hours duration. Cold shock activation for 2 hours provides a recipient oocyte for fusion that is in S-phase and about 9 hours away from its next M-phase. The donor embryonic cells used in Example 2, with a cell cycle time of about 20 hours, were cultured for about 10 hours after removal of the Nocodazole, to provide a donor cell that is in S-phase and about 10 hours away from its next M-phase.

Blastomeres obtained from mid-stage embryos and fused to activated recipient oocytes yielded NT embryos having an enhanced ability to mature to the blastocyst stage *in vitro* when compared to NT embryos produced using non-synchronized donor blastomeres. NT embryos produced from synchronized blastomeres reached the blastocyst stage 27% of the time while NT embryos derived from non-synchronized donors reached the blastocyst stage only 18% of the time.

The NT data of this example demonstrate that development is better when the donor nucleus and the recipient oocyte are both in S-phase (or mid-stage) and would both reach the next mitotic division at the same time.

Example 3

Synchronization of Nocodazole-treated Bovine Embryos and Cold-Culture-Activated Recipient Oocytes Matured for 24 Hours In Vitro

Recipient oocytes were matured *in vitro* as described above for 20-28 hours before activation. Fusion with synchronized donor cells was performed approximately 6 hours post-activation. Oocytes that have matured *in vitro* for 20-28 hours have a cell cycle time of approximately 24 hours. Therefore, these activated recipient oocytes were approximately 18 hours from next metaphase.

To demonstrate the benefit of donor-recipient synchronization, bovine blastomere cells in two different portions of S-phase were used as donors in fusions with the above-described activated oocytes. Bovine embryo cells

were blocked in metaphase with 1.0 μM Nocodazole and were then allowed to proceed into the cell cycle for 5 or 10 hours post-removal of the metaphase block. At five hours, the donor blastomeres had passed through G1-phase and were in early S-phase. The donor blastomeres were approximately 15-18 hours away from next metaphase. By 10 hours, the blastomeres were in mid-S-phase and were about 10 hours away from next metaphase.

Fusions were performed as detailed above. Table 3 details the results of fusions with donor cells that were either synchronized or unsynchronized with recipient oocytes with respect to the next metaphase. It is apparent from the data that when the time to next metaphase is similar, the percentage of NT embryos that develop to the blastocyst stage is markedly higher.

TABLE 3

Synchronized Oocytes		Synchronized Blastomeres		Percentage of Blastocysts Obtained (Blastocysts/ Total NT Embryos)
Time since last meta-phase	Time until next meta-phase	Time since last meta-phase	Time until next meta-phase	
6	18	5	15	27% (9/33)
		10	10	2% (3/134)

CLAIMS

We claim:

1. A method for preparing donor bovine embryonic cells for a nuclear transfer process comprising the steps of:

(a) isolating bovine embryonic cells;

(b) synchronizing the isolated embryonic cells until a majority of the cells are at a single mitotic phase;

(c) culturing the synchronized embryonic cells of step (b) until at least some of the synchronized embryonic cells are in the S-phase of the cell cycle; and

(d) selecting an individual embryonic cell from the culture of step (c) for use in a bovine nuclear transfer process.

2. A method for preparing donor bovine embryonic cells for a nuclear transfer process as claimed in claim 1 wherein synchronizing step (b) is accomplished by culturing the embryonic cells in the presence of a cell cycle inhibitor for a length of time, and at a concentration, sufficient to reversibly arrest the cell cycle at the single mitotic phase.

3. A method for preparing donor bovine embryonic cells for a nuclear transfer process as claimed in claim 2 wherein synchronizing step (b) leaves the bovine embryonic cells synchronized in metaphase.

4. A method for preparing donor bovine embryonic cells for a nuclear transfer process as claimed in claim 3 wherein synchronizing step (b) is performed by culturing the embryonic cells in Demecolcine.

5. A method for preparing donor bovine embryonic cells for a nuclear transfer process as claimed in claim 4 wherein synchronizing step (b) is performed by culturing the embryonic cells in Demecolcine for 12-18 hours at a concentration of 0.05 µg/ml.

6. A method for preparing donor bovine embryonic cells for a nuclear transfer process as claimed in claim 3 wherein synchronizing step (b) is performed by culturing the embryonic cells in Nocodazole.

7. A method for preparing donor bovine embryonic cells for a nuclear transfer process as claimed in claim 6 wherein synchronizing step (b) is performed by culturing the embryonic cells in Nocodazole for 12-18 hours at a concentration of 1.0 µM.

8. A method for preparing donor bovine embryonic cells for a nuclear transfer process as claimed in claim 1 wherein the culturing step (e) proceeds for 8-14 hours post-metaphase.

9. A method for producing a multiplied bovine embryo comprising the steps of:

(a) isolating a recipient bovine oocyte;

(b) enucleating the recipient oocyte;

(c) isolating bovine donor cells;

(d) synchronizing a majority of the donor cells at a single mitotic phase;

(e) culturing the synchronized donor cells of step (d) until at least some of the donor cells are in the S-phase of the mitotic cell cycle;

(f) selecting an individual donor cell from the donor cell culture of step (e);

(g) introducing the individual donor cell of step (f) into the perivitelline space of the enucleated oocyte. the individual donor cell and the enucleated oocyte being selected so that they have approximately equal time-until-next-M-phase;

(h) fusing the donor cell into the recipient oocyte to make a nuclear transfer embryo; and

(i) transferring the nuclear transfer embryo into a maternal animal.

10. A method for producing a multiplied bovine embryo as claimed in claim 9 wherein synchronizing step (d) is accomplished by culturing the donor cells in the presence of a cell cycle inhibitor for a length of time, and at a concentration, sufficient to reversibly arrest the cell cycle at the single mitotic phase.

11. A method for producing a multiplied bovine embryo as claimed in claim 10 wherein synchronizing step (d) leaves the bovine donor cells synchronized in metaphase.

12. A method for producing a multiplied bovine embryo as claimed in claim 11 wherein synchronizing step (d) is performed by culturing the donor cells in Demecolcine.

13. A method for producing a multiplied bovine embryo as claimed in claim 12 wherein synchronizing step (d) is performed by culturing the donor cells in Demecolcine for 12-18 hours at a concentration of 0.05 µg/ml.

14. A method for producing a multiplied bovine embryo as claimed in claim 11 wherein synchronizing step (d) is performed by culturing the donor cells in Nocodazole.

15. A method for producing a multiplied bovine embryo as claimed in claim 14 wherein synchronizing step (d) is performed by culturing the donor cells in Nocodazole for 12-18 hours at a concentration of 1.0 µM.

16. A method for producing a multiplied bovine embryo as claimed in claim 9 wherein the culturing step (e) proceeds for 8-14 hours post-metaphase.

17. A method for producing a multiplied bovine embryo as claimed in claim 9 further comprising the step of activating the recipient oocyte before fusion.

18. A method for producing a multiplied bovine embryo as claimed in claim 17 wherein the recipient oocyte is activated by culturing the recipient oocyte at a temperature of less than 30°C for at least two hours before fusion.

19. A method for producing a multiplied bovine embryo as claimed in claim 18 wherein synchronizing step (d) is accomplished by culturing the donor cells in the presence of a cell cycle inhibitor for a length of time, and at a concentration, sufficient to reversibly arrest the cell cycle at the single mitotic phase.

20. A method for producing a multiplied bovine embryo as claimed in claim 19 wherein synchronizing step (d) leaves the bovine donor cells synchronized in metaphase.

21. A method for producing a multiplied bovine embryo as claimed in claim 20 wherein synchronizing step (d) is performed by culturing the donor cells in Nocodazole.

22. A method for producing a multiplied bovine embryo as claimed in claim 21 wherein synchronizing step (d) is performed by culturing the donor cells in Nocodazole for 12-18 hours at a concentration of 1.0 μM.

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23. A method for producing a multiplied bovine embryo as claimed in claim 9 wherein the recipient oocyte is activated by culturing the recipient oocyte at a temperature of less than 30°C for at least two hours before fusion.

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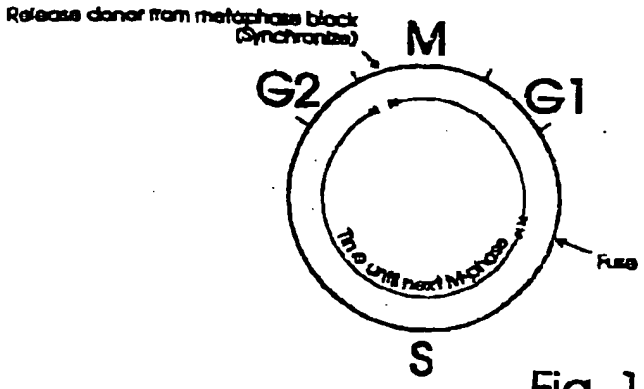

Fig. 1

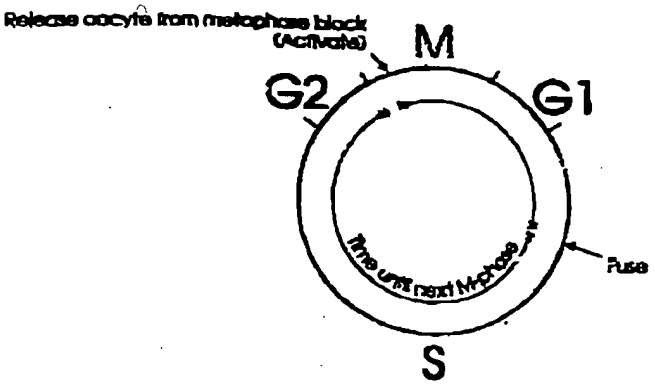

Fig. 2

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